

genomic hybridization and found that genomic profiles are indistinguishable between adenocarcinoma *in situ* and minimally invasive adenocarcinoma, although they developed in distinct individuals (5). In contrast, this study successfully revealed that gene expression profiles substantially differed between lepidic growth and microinvasion cancer cells in a single minimally invasive adenocarcinoma. The findings of these two studies and the present results indicate that the Notch2 and Six1 upregulation detected in minimally invasive adenocarcinoma is attributable not to particular genomic alterations, but to environmental factors, including immune and stromal cells around cancer cells. In fact, activation of the Notch2 signaling pathway is cell membrane-bound ligand dependent (26). Although further characterization of lepidic-predominant invasive adenocarcinoma is required at the molecular level, subclassification of lepidic-predominant invasive adenocarcinoma on the basis of Notch2 and Six1

upregulation seems to be clinically important in predicting the prognostic outcomes of patients with lepidic-predominant invasive adenocarcinoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Tumor suppressor cell adhesion molecule 1 (CADM1) is cleaved by a disintegrin and metalloprotease 10 (ADAM10) and subsequently cleaved by γ -secretase complex

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ABSTRACT

Cell adhesion molecule 1 (CADM1) is a type I transmembrane glycoprotein expressed in various tissues. CADM1 is a cell adhesion molecule with many functions, including roles in tumor suppression, apoptosis, mast cell survival, synapse formation, and spermatogenesis. CADM1 undergoes membrane-proximal cleavage called shedding, but the sheddase and mechanisms of CADM1 proteolysis have not been reported. We determined the cleavage site involved in CADM1 shedding by LC/MS/MS and showed that CADM1 shedding occurred in the membrane fraction and was inhibited by tumor necrosis factor- α protease inhibitor-1 (TAPI-1). An siRNA experiment revealed that ADAM10 mediates endogenous CADM1 shedding. In addition, the membrane-bound fragment generated by shedding was further cleaved by γ -secretase and generated CADM1-intracellular domain (ICD) in a mechanism called regulated intramembrane proteolysis (RIP). These results clarify the detailed mechanism of membrane-proximal cleavage of CADM1, suggesting the possibility of RIP-mediated CADM1 signaling.

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1. Introduction

Cell adhesion molecule 1 (CADM1) is a 100–120-kDa multifunctional cell adhesion molecule. CADM1 is a member of the immunoglobulin superfamily and is a type I transmembrane glycoprotein. The extracellular domain of CADM1 undergoes homophilic or heterophilic interaction with necl-1, nectin-3, and CRTAM (class-I MHC-restricted T cell-associated molecule) [1], whereas the intracellular domain interacts with DAL-1 via its band 4.1 binding domain [2] and with pals2 [3] and MPP3 [4] via its PDZ binding domain. CADM1 is expressed in various tissues and organs, including the brain, mast cells, testis, and lung [5].

Recently, Tanabe et al. reported that CADM1 undergoes juxtamembrane cleavage in neurons [6]. We reported previously that shedding fragments were found from mast cells [7] and human mesothelioma samples [8]. These observations suggest that CADM1

shedding is a common event. CADM1 shedding seems to regulate adhesion between mesothelial and mesothelioma cells by down-regulating full-length CADM1, thereby regulating the growth and scattering of mesothelioma cells [8]. Tanabe et al. reported that certain isoforms of CADM1 produce a 20-kDa C-terminal fragment and that production of this fragment is inhibited by the broad-spectrum metalloprotease inhibitor TAPI-1. They concluded that CADM1 is shed by an ADAM17-like protease [6]. The ADAM family metalloproteases are the major membrane-bound sheddases, which are responsible for shedding of many membrane protein substrates. Among the family members, ADAM10 and ADAM17 are the major sheddases and have large numbers of known substrates. After shedding, some membrane proteins undergo a secondary cleavage within the membrane mediated by γ -secretase. For example, Notch, ErbB4, and amyloid precursor protein (APP) are cleaved by γ -secretase [9]. The γ -cleavage product, the intracellular domain (ICD) of the substrate, often acts as a transcriptional regulator. ICDs of Notch and other γ -secretase substrates, such as CD44 [10] and APP [11], act as nuclear signaling molecules that regulate gene expression.

In this study, we focused on CADM1 proteolysis. The aim of this study was to characterize the sheddase of CADM1 and to examine proteolysis events occurring on the CADM1 molecule. Our results

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indicated that CADM1 shedding is catalyzed by a protease associated with the cell membrane, and ADAM10 endogenously sheds CADM1. Furthermore, we discovered γ -cleavage of CADM1, which is catalyzed by γ -secretase. These results provide a possible signal-transduction mechanism involving regulated intramembrane proteolysis (RIP).

2. Materials and methods

2.1. Reagents and antibodies

TAPI-1, *N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-(*S*)-phenylglycine *t*-butyl ester (DAPT), and L-685,458 were purchased from Calbiochem (San Diego, CA). Phorbol-12-myristate-13-acetate (PMA) was purchased from Wako (Osaka, Japan). Phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail (cat.# P8340) were purchased from Sigma (St. Louis, MO).

Anti-CADM1-cyto antibody was generated according to the method described by Wakayama et al. [12]. This antibody recognizes the C-terminal 20 amino acids of CADM1. Anti-CADM1-ecto antibody (3E1) is our original as described previously [13]. This antibody recognizes 2nd Ig domain in the ectodomain. Peroxidase-conjugated secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA).

2.2. In vitro assay of CADM1 shedding

Confluent HEK293 cells were harvested and homogenized in buffer P (20 mM PIPES-KOH pH 7.0, 140 mM KCl, 250 mM sucrose) at 4 °C. The homogenized cells were centrifuged at 800g for 10 min to remove the nuclei and cell debris. The supernatant was then ultracentrifuged at 100,000g for 1 h, and the resulting pellet was washed with buffer P and ultracentrifuged again at 100,000g for 1 h. The resulting pellet (the membrane fraction) was resuspended in buffer P and stored at –80 °C until use.

This membrane fraction was incubated at 37 °C for 1.5 h with the indicated concentrations of protease inhibitors or vehicle only (DMSO, 0.2% for TAPI-1, 1% for PMSF and protease inhibitor cocktail), and the reaction was stopped by addition of 2× sample buffer and boiling for 5 min. These samples were subjected to Western blotting analysis using anti-CADM1-cyto antibody.

2.3. Western blotting analysis

SDS-PAGE and Western blotting were performed according to the standard reducing SDS-PAGE and Western blotting protocols. For detection of CADM1-ICD, samples were separated by Tris-tricine SDS-PAGE and transferred onto Immobilon-P membranes (Millipore, Billerica, MA). The membranes were boiled in PBS for 5 min after transfer and then subjected to blocking, antibody treatments, and subsequent procedures.

2.4. Transfection of siRNA and expression vectors

siRNA (Stealth Select RNAi) specific for human ADAM10 (HSS100167), ADAM17 (HSS110435), and control siRNA (Stealth RNAi negative control low GC duplex, 12935-200) were purchased from Invitrogen (Carlsbad, CA). Saos-2 cells at 40–50% confluence were transfected with siRNAs using Lipofectamine RNAiMax Transfection Reagent (Invitrogen) according to the manufacturer's instructions. After 96 h of incubation, cells were harvested, and the cell lysates were analyzed by Western blotting.

2.5. Cell culture

cDNA encoding mouse-CADM1 (GenBank ID: AB052293) was cloned into the expression vector pCX4-bsr and confirmed by DNA sequencing (pCX4bsr-CADM1) [7]. COS7 cells and NIH3T3 cells were transfected with the pCX4bsr-CADM1 vector, and blasticidin-resistant cells were selected by continuing the culture in the presence of blasticidin (3 μ g/mL) for 4 weeks (COS7-mCADM1 and NIH3T3-mCADM1). MEF and nicastrin^{−/−} MEF cells were kind gifts from Dr. Philip C. Wong (The Johns Hopkins University), and presenilin 1/2 double knockout MEF was a kind gift from Dr. Bart De Strooper (Vlaams Instituut voor Biotechnologie). These cells, HEK293, and human osteosarcoma Saos-2 were maintained in DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin. In γ -secretase inhibitor assay, confluent COS7-mCADM1 cells were treated with 200 ng/mL PMA for 2 h, and then γ -secretase inhibitor (1 μ M DAPT or 1 μ M L-685,458) or vehicle (DMSO) was added. After incubation for the indicated times, cells were harvested, and the cell lysates were analyzed by Western blotting.

2.6. Mass spectrometric analysis

NIH3T3-mCADM1 cells were harvested, and the membrane fraction extract was prepared using Subcellular Proteome Extraction Kit (Merck, Whitehouse Station, NJ). The membrane fraction extract was concentrated using Amicon Ultra 15 (Millipore) and then immunoprecipitated using anti-CADM1cyto antibody. The precipitate was mixed with sample buffer and subjected to SDS-PAGE. The gel was stained with EZ-Blue (Sigma), and the alphaCTF band was cut out. In-gel digestion with chymotrypsin was performed according to the published methods [14,15]. The digested peptides were subjected to liquid chromatography (LC)/MS/MS analysis on a Q-ToF2 quadrupole/time-of-flight (TOF) hybrid mass spectrometer (Micromass, Manchester, UK) interfaced with a capillary reversed-phase liquid chromatography system (Micromass CapLC system) as described previously [14]. The MS/MS data were analyzed with Mascot MS/MS Ion Search (version 2.1.6; Matrix Science Ltd., London, UK) to assign the obtained peptides to the NCBI non-redundant database (NCBI nr 20060718; 3784285 sequences) as described previously [14].

3. Results

3.1. CADM1 shedding is promoted by phorbol ester

To study shedding of CADM1, we first generated a stable COS7 cell line expressing murine CADM1 (COS7-mCADM1). Several CADM1-derived bands, including bands of approximately 15 and 35 kDa, were detected from COS7-mCADM1 lysate by Western blotting with an antibody that recognizes the C-terminal of CADM1. We refer to the 15- and 35-kDa fragments as alphaCTF (C-terminal fragment) and betaCTF, respectively. AlphaCTF is likely to be the same shedding fragment of CADM1 reported previously [6], and betaCTF is another fragment of CADM1. As shedding of some other membrane proteins is promoted by phorbol esters such as PMA, we examined the effects of PMA treatment on CADM1 shedding (Fig. 1A) and found that alphaCTF was increased after PMA treatment. On the other hand, no apparent changes were observed in the 35-kDa band, which was thought to be another cleaved fragment of CADM1.

We next concentrated CADM1 alphaCTF by immunoprecipitation and determined the cleavage site by LC/MS/MS. Following SDS-PAGE, the gel slice containing the alphaCTF band was excised

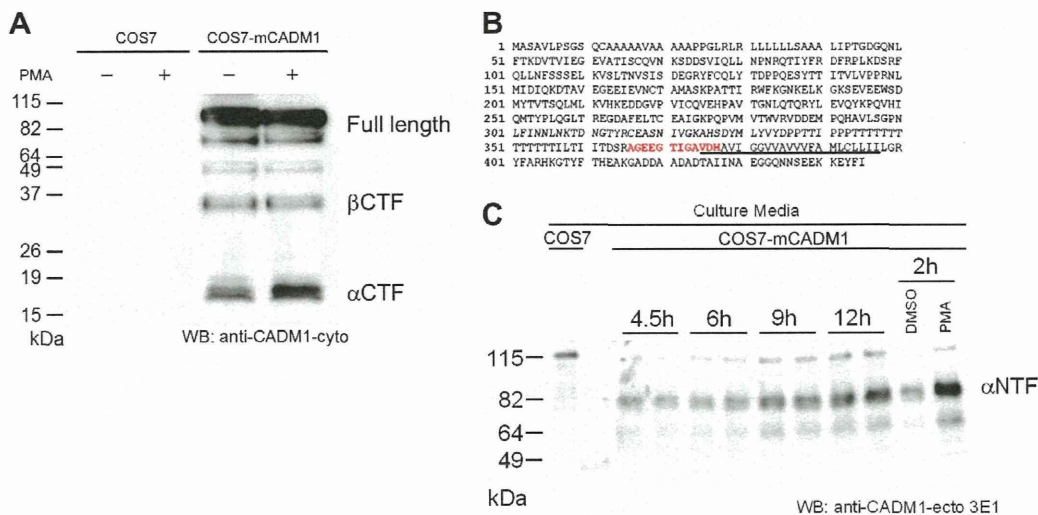


Fig. 1. Ectodomain shedding of CADM1. (A) COS7-mCADM1 cells were treated with 200 ng/mL PMA or vehicle alone for 30 min and then harvested. Cell lysates were analyzed by Western blotting using anti-CADM1-cyto antibody. (B) Determination of the cleavage site of CADM1 shedding. The peptide detected by LC/MS/MS is indicated in red, and the transmembrane region is underlined. (C) Culture medium of subconfluent COS7-mCADM1 cells was changed to serum-free medium and incubated for the indicated times (duplicate results are shown). To evaluate PMA-dependent secretion, 200 ng/mL PMA or vehicle (DMSO) was added to the serum-free medium and incubated for 2 h. The medium was then harvested and concentrated by TCA precipitation. The precipitate was analyzed by Western blotting using anti-CADM1-ecto antibody 3E1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and cleaved with chymotrypsin in the gel. LC/MS/MS detected a peptide fragment with an N-terminus generated by proteases other than chymotrypsin (Fig. 1B). This observation suggested that the N-terminus of the detected peptide was generated by shedding. The cleavage site was between R365 and A366, nine amino acid residues from the predicted transmembrane region in the extracellular domain. This result predicted that alphaCTF is an 80 amino acids fragment. Larger apparent molecular weight of this fragment on Western blotting is probably due to O-linked glycosylation. This result also predicted that N-terminal fragments are released into the culture medium after shedding and that PMA treatment would increase this release. Therefore, we analyzed the culture media by Western blotting and found that there was indeed a CADM1 fragment corresponding in size to NTF after cleavage that produces alphaCTF. As expected, we also found that PMA treatment increased the amount of CADM1 fragments in the culture medium (Fig. 1C).

Taken together, these results suggest that CADM1 shedding produces alphaCTF and a corresponding extracellular fragment, that cleavage occurs in the membrane-proximal region of CADM1 extracellular domain, and that shedding is promoted by PMA.

3.2. CADM1 shedding was detected in an *in vitro* membrane-fraction incubation assay

CADM1 shedding was previously shown to be inhibited by TAPI-1, a broad-spectrum inhibitor that targets many metalloproteases such as ADAMs and MMPs. Among them, those with transmembrane domains are known to shed a large number of transmembrane substrates.

To obtain further information about CADM1 sheddase, we performed an *in vitro* assay using total cell membrane fraction and focusing on membrane-bound proteases.

To avoid artifacts due to overexpression of CADM1, we prepared total membrane fraction from HEK293 cells, which constitutively express endogenous CADM1. The total membrane fraction prepared by ultracentrifugation was incubated. The level of alphaCTF increased after incubation, whereas that of full-length CADM1 decreased (Fig. 2A). BetaCTF was barely detectable in this prepara-

tion. The increase in the 15-kDa band was inhibited by addition of TAPI-1 (Fig. 2A and B), as reported previously by Tanabe et al. [6], who added TAPI-1 to neuronal cells. Serine, cysteine, and aspartic protease inhibitors showed no inhibitory effect on CADM1 shedding (Fig. 2C). Thus, we could reconstitute shedding of CADM1 *in vitro* by incubation of the membrane fraction and could characterize the sensitivity to protease inhibitors using this method. These results indicated that a membrane-associated metalloprotease possesses CADM1 shedding activity.

3.3. CADM1 shedding is mediated by the metalloprotease ADAM10

Many membrane molecules undergo shedding, and ADAM10 and ADAM17 were reported to be two major membrane-associated sheddases. An siRNA approach was employed here to investigate whether ADAM10 and/or ADAM17 cleaved CADM1. The Saos-2 human osteosarcoma cell line, which shows high-level expression of endogenous ADAM10, was used in this assay. The siRNAs used downregulated the mature form of targeted ADAMs as well as the proform (Fig. 3A). Knockdown of ADAM10 expression decreased the amount of alphaCTF, whereas the knockdown of ADAM17 had no effect on the amount of alphaCTF. Furthermore, ADAM10 knockdown abolished PMA-dependent accumulation of alphaCTF (Fig. 3B). These observations indicated that CADM1 is cleaved endogenously by the metalloprotease ADAM10, and PMA-dependent cleavage of CADM1 is also mainly mediated by ADAM10.

3.4. γ -Secretase-like cleavage is abrogated in the presence of γ -secretase inhibitors and in γ -secretase KO cells

Following membrane-proximal cleavage, similar to CADM1 shedding, some membrane proteins undergo a second cleavage mediated by γ -secretase. To determine whether CADM1 is cleaved by γ -secretase, we examined the effects of γ -secretase inhibition on the amounts of cleaved CADM1 fragments after induction of shedding by PMA. COS7-mCADM1 cells were treated with PMA or vehicle alone and then treated with γ -secretase inhibitor (DAPT or L-685,458) or vehicle alone. The amount of alphaCTF increased in

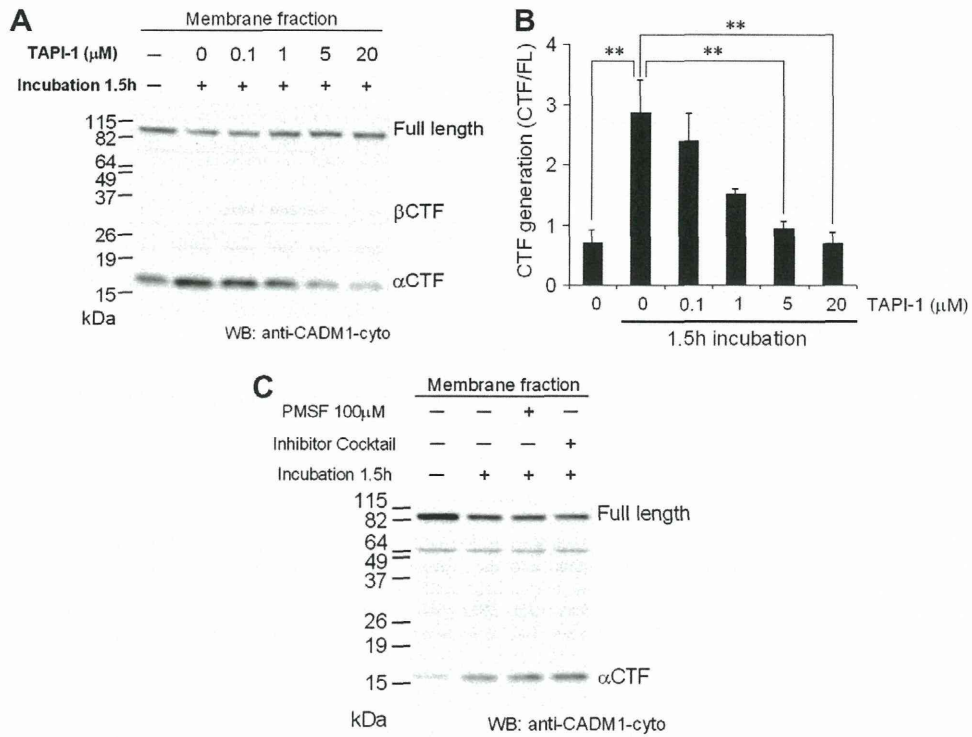


Fig. 2. *In vitro* CADM1 shedding assay. (A–C) Membrane fraction was obtained from HEK293 cells as described in Section 2. The membrane fraction was incubated with the indicated concentration of protease inhibitors and subjected to Western blotting analysis using anti-CADM1-cyto antibody. The results of densitometric analysis of CADM1-CTF generation in (A) are shown in (B). CTF generation was calculated as CTF/full-length ratio by densitometric analysis. Results were obtained from three independent experiments and are expressed as means ± SEM. Data were analyzed by one-way analysis of variance followed by Dunnett's multiple comparison test. **Statistically significant compared with "1.5 h, 0 μM TAPI-1" ($p < 0.01$). The inhibitor cocktail in (C) was from Sigma (P8340).

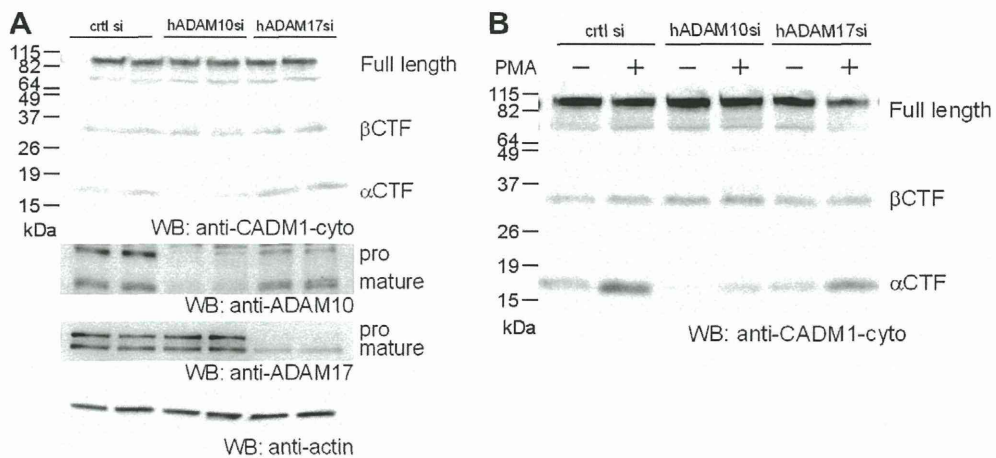


Fig. 3. Knockdown of ADAM10 decreases CADM1-CTF level. (A) Saos-2 cells were transfected with siRNAs against ADAM10 or ADAM17 or with control siRNA. Cells were harvested after 96 h of incubation. Cell lysates were subjected to Western blotting analysis using anti-CADM1-cyto antibody (duplicate results are shown). In (B), cells were treated with 200 ng/mL PMA or vehicle only (DMSO) for 4 h and then harvested.

the presence of γ -secretase inhibitor (Fig. 4A), indicating γ -secretase-dependent degradation of alphaCTF. This finding suggested that alphaCTF was cleaved by γ -secretase into a cytosolic fragment, CICD (CADM1 intracellular domain). With membrane boiling and prolonged exposure, a CADM1-derived band of nearly 6 kDa was detected (Fig. 4A). Membrane boiling contributes to the detection of this 6 kDa fragment, and it is necessary to boil the membrane for the detection of obvious CICD band. The amount of this fragment was decreased with γ -secretase inhibitor treatment, consistent with alphaCTF accumulation (Fig. 4A). γ -Secretase cleaves its sub-

strates within the cell membrane at around three amino acids from the cytosolic end of the transmembrane region of the substrate and generates a fragment with an intact cytoplasmic domain. The theoretical molecular weight of CADM1 cytoplasmic domain is 5.3 kDa. This fragment is the cleavage product of CADM1 generated by γ -secretase. These results suggest that alphaCTF is cleaved by γ -secretase and generates CICD fragments.

We employed another approach to further confirm this possibility. γ -Secretase consists of four proteins, presenilin, nicastrin, Aph-1, and Pen-2, all of which are indispensable for its activity. We used

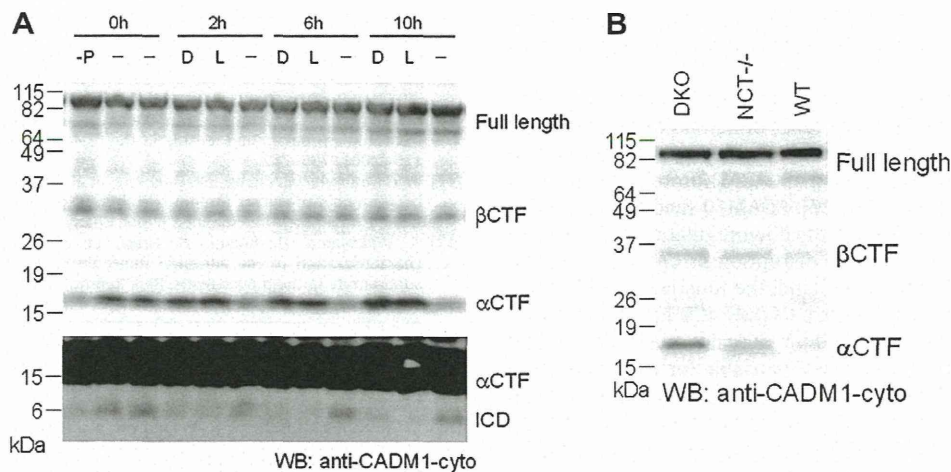


Fig. 4. CADM1-ICD generation is dependent on γ -secretase. (A) COS7-mCADM1 cells were pretreated with 200 ng/mL PMA or vehicle alone (–P: DMSO) for 2 h, and subsequently treated with γ -secretase inhibitors (D: 1 μ M DAPT or L: 1 μ M L-685,458) or vehicle alone (–: DMSO) and incubated for the indicated times. (B) Cultured cells of two mouse embryonic fibroblast (MEF) cell lines lacking γ -secretase activity and a wild-type MEF cell line were harvested. Cell lysates were analyzed by Western blotting using anti-CADM1-cyto antibody.

two MEF cell lines with no γ -secretase activity, one lacking presenilin-1 and presenilin-2 (PSDKO) and another lacking nicastrin (NCT^{-/-}) [16,17]. On Western blotting analysis, the alphaCTF level was increased in γ -secretase KO MEFs but not in wild-type MEFs (Fig. 4B). These results were consistent with those of γ -secretase inhibitor assay, indicating that alphaCTF is processed into CICD by γ -secretase.

4. Discussion

Many previous studies have addressed the various functions of CADM1. Genetic analyses of human cancer suggest that CADM1 has potent tumor-suppressor activity. CADM1 was also shown to suppress tumorigenicity in mouse models, to induce apoptosis, and to suppress cell proliferation *in vitro* [18]. However, the molecular mechanisms underlying these and other functions are largely unknown. In the present study, we focused on membrane protein proteolysis, which may modulate or mediate the functions of such proteins. A previous study indicated that certain CADM1 isoforms are shed due to the action of a ADAM17-like protease [6]. We examined CADM1 proteolysis in more detail, and here we demonstrated the two-step mechanism of CADM1 proteolysis including shedding and RIP with identification of the cleavage site of shedding, sheddase, and subsequent γ -cleavage.

We first demonstrated enhancement of CADM1 shedding by PMA and accumulation of the cleaved extracellular fragment in the medium. A soluble CADM1 isoform was shown to elicit directional neurite extension up a CADM1 concentration gradient [19]. According to the cleavage site determined here, the whole sequence of the soluble isoform was contained within the shed fragment with the exception of two amino acids near the isoform-specific C-terminus. These observations suggest that CADM1 extracellular fragment may also serve as a neurite attractant with a regulated mechanism of secretion. Unexpectedly, although betaCTF was abundant in several cell lines, betaNTF was much less abundant than alphaNTF in the culture medium of the same cells. An as-yet-unknown cellular system may retain and degrade the “extracellular” fragment within the cells.

We developed an *in vitro* shedding assay and demonstrated that CADM1 shedding could occur not only in the intact cell but also in the isolated membrane fraction. This method can be used as a novel *in vitro* assay for ectodomain shedding. To date, *in vitro* shedding assays have mostly been performed by the addition of

purified protease to the membrane fraction, which contains the substrate. Our method is closer to physiological conditions because the endogenous localization of a protease and its substrate should be intact. Using this method, we showed here that shedding of CADM1 was directly mediated by a membrane-bound metalloprotease. TAPI-1 was also shown to have an inhibitory effect on CADM1 shedding, consistent with previous findings [6]. Furthermore, the results of siRNA experiments indicated that ADAM10, but not ADAM17, is a potent endogenous sheddase of CADM1. In contrast, previous studies showed that nectin-1 and nectin-4, both of which are adhesion molecules related to CADM1, are cleaved by ADAM10 and ADAM17, respectively [20,21]. ADAM10 is a well-known sheddase that cleaves many other adhesion molecules. Although no consensus cleavage sequence is known for ADAM10, this molecule cleaves its substrate at a site 8–20 amino acids from the transmembrane region in its ectodomain [22]. In the present study, the cleavage site for CADM1 shedding was determined to be nine amino acids from the transmembrane region by MS analysis, indicating that shedding of CADM1 is similar to that of other known cell-surface molecules mediated by ADAM10.

We next showed that CADM1 undergoes γ -secretase-mediated cleavage and generates CADM1-ICD using inhibitors or γ -secretase-deficient cell lines. A previous study indicated that nectin-1 α is also cleaved by γ -secretase-like activity to generate ICD [23]. This process is known to involve proteolytic cleavage called RIP, in which removal of the ectodomain by shedding is necessary for the second cleavage catalyzed by γ -secretase [9]. Our data suggested that the substrate of γ -cleavage is the membrane-bound shedding product alphaCTF, consistent with the known mechanism of RIP. Compared with alphaCTF, the amount of CICD fragment is very small, and the difference between the amount of the fragment with and without inhibitor treatment was small. This is probably because of the rapid degradation of CICD, as many other ICD fragments generated by γ -secretase, including Notch ICD, are known to be rapidly degraded in a proteasome-dependent manner [23].

ICD often acts as a signal transducer. Nuclear staining for CADM1 was observed on microscopic analysis using C-terminal GFP-tagged CADM1 or antibody against CADM1 cytosolic domain ([1] and unpublished data). CASK, a protein that binds to CADM1 cytoplasmic domain, is partially localized to the nucleus and interacts with the T-box transcription factor Tbr-1, inducing transcription of genes including NR2b [24]. CADM1-ICD may be transported into the nucleus together with CASK and may modulate

target genes of the Tbr-1-CASK complex. These observations suggest that CADM1 may function in the nucleus as a signal transducer via RIP.

CADM1 is known to act as a tumor suppressor in nude mice, and its expression is reduced in many types of human tumor. ADAM10 is overexpressed in colorectal cancer [25]. The hypermethylation of CADM1 and the reduced expression of CADM1 protein in colorectal cancer have also been reported [26]. ADAM10-mediated shedding may be a mechanism involved in the downregulation of CADM1 in colorectal cancer, which leads to disruption of CADM1-mediated tumor suppression. On the other hand, the functional significance of γ -cleavage is rather complicated. CADM1-ICD has two protein-binding domains, band 4.1 binding domain and PDZ binding domain [1]. These domains are indispensable for the tumor-suppressive functions of CADM1, including tumor suppression in mice, the induction of apoptosis, and the suppression of epithelial-mesenchymal transition [27–29], suggesting the functional significance of the CADM1 cytoplasmic domain.

In conclusion, our data demonstrated that CADM1 cleavage is mediated by ADAM10 and γ -secretase. This mechanism may be important for the downregulation of CADM1 by proteolytic degradation or for tumor-suppressive activity and other functions of CADM1, probably through the activation of CADM1-ICD and reduction of cell–cell adhesion.

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Aberrant expression of tumor suppressors *CADM1* and *4.1B* in invasive lesions of primary breast cancer

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Abstract

Background The tumor suppressor genes *CADM1/TSLC1* and *DAL-1/4.1B* are frequently inactivated by promoter methylation in non-small cell lung cancer. The proteins they encode, *CADM1* and *4.1B*, form a complex in human epithelial cells and are involved in cell–cell adhesion.

Methods Expression of *CADM1* and *4.1B* proteins was examined by immunohistochemistry in 67 primary breast cancer and adjacent noncancerous tissues. *CADM1* and *4.1B* messenger RNA (mRNA) was detected by reverse-transcription polymerase chain reaction (RT-PCR). The methylation status of the *CADM1* and *4.1B* promoters was determined quantitatively by bisulfite treatment followed by pyrosequencing.

Results *CADM1* and *4.1B* protein signals were detected along the cell membrane in normal mammary epithelia. By contrast, 47 (70%) and 49 (73%) of 67 primary breast cancers showed aberrant *CADM1* and *4.1B* staining, respectively. Aberrant *CADM1* staining was more frequently observed in pT2 and pT3 tumors and for stages II

and III ($P = 0.045$ and $P = 0.020$, respectively), while aberrant *4.1B* staining was more often observed in tumors with lymph node metastasis, for pT2 and pT3 tumors, and for stages II and III ($P = 0.0058$, $P = 0.0098$, and $P = 0.0007$, respectively). Furthermore, aberrant *CADM1* and *4.1B* expression was preferentially observed in invasive relative to noninvasive lesions from the same specimen ($P = 0.036$ and $P = 0.0009$, respectively). Finally, hypermethylation of *CADM1* and *4.1B* genes was detected in 46% and 42% of primary breast cancers, respectively.

Conclusions Our findings suggest that aberrant *CADM1* and *4.1B* expression is involved in progression of breast cancer, especially in invasion into the stroma and metastasis.

Keywords *CADM1* · *4.1B* · Tumor suppressor protein · Breast cancer · Methylation

Introduction

Breast cancer is the most common malignancy in women, and its incidence has been increasing in recent years in Japan. Many clinical and pathological factors are routinely used to categorize patients with breast cancer in order to assess prognosis and determine the most appropriate therapy. Patient age, tumor size, lymph node metastasis, nuclear grade, lymphovascular invasion, expression status of hormone receptors including the estrogen receptor (ER) and progesterone receptor (PgR), and status of human epidermal growth factor receptor 2 (Her2) have been recognized as major prognostic factors. Although these factors are mostly useful, more effective indicators are required to determine prognosis precisely and to evaluate the risk of recurrence in patients with breast cancer. Recently,

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additional new techniques, such as gene expression profiling [1], have been developed to improve subclassification of breast cancer and to determine the most appropriate therapy as well. However, these mRNA-based diagnostic approaches have several disadvantages in practical use.

The tumor suppressor gene *CADM1* (*Cell adhesion molecule 1*)/*TSLC1* (*Tumor suppressor in lung cancer 1*) was identified on chromosome 11q23.2 by functional complementation of tumorigenicity of non-small cell lung cancer (NSCLC) cells [2–5]. *CADM1* encodes an immunoglobulin superfamily molecule that is involved in cell–cell adhesion in a variety of human epithelia, including those of the mammary gland [5]. Previous studies have revealed that *CADM1* is frequently inactivated in various cancers, such as lung [2, 3], prostate [6], liver, pancreas [4], and breast cancer [7], especially in those with invasion and metastasis to lymph nodes and distant organs. Goto et al. [15] reported that *CADM1* expression was preferentially lost in invasive lesions relative to noninvasive lesions of lung adenocarcinoma. Hypermethylation of the *CADM1* promoter has been shown to be one of the main mechanisms to inactivate the gene in these cancers [2–4, 6–8].

The tumor suppressor gene *DAL-1/4.1B* (*differentially expressed in adenocarcinoma of the lung*) was shown to be located on chromosomal fragment 18p11.3 and was inactivated in lung, breast, and brain tumors [9, 10]. The protein it encodes, 4.1B, belongs to the protein 4.1 superfamily of scaffold proteins. Yageta et al. [10, 11] reported that *CADM1* interacts with the actin filament through 4.1B at the cell–cell attachment site, where complex formation of *CADM1* and 4.1B is dependent on the integrity of the actin cytoskeleton, and that *CADM1* and 4.1B are responsible for stable adhesion between adjacent cells. It has also been reported that loss of 4.1B expression and methylation of the *4.1B* promoter are involved in development and progression of NSCLC, providing a possible indicator of poor prognosis [12]. Thus, the *CADM1*–4.1B cascade appears to be involved in cell–cell attachment, while functional loss of *CADM1* and/or 4.1B would play a role in invasion and metastasis of tumor cells in advanced stages.

In breast cancer, promoter methylation of *CADM1* was found in 33% of surgically resected tumors using bisulfate sequencing analysis [7]. Furthermore, loss of 4.1B expression was observed in 83% of breast cancer cell lines using Western blotting [13]. Previous studies have also shown that *CADM1* and/or *4.1B* methylation correlates with tumor grade and hormone receptor status in breast cancer [14].

In the present study, we examined expression of *CADM1* and 4.1B in 67 primary breast cancer specimens and the methylation status of *CADM1* and *4.1B* promoters in 6 breast cancer cell lines and 50 primary breast cancers. We then examined whether expression of *CADM1* and

4.1B as well as their methylation status correlated with the clinicopathological factors of primary breast cancers in order to investigate the significance of *CADM1* and 4.1B in breast tumorigenesis. Our results suggest that loss of *CADM1* and 4.1B expression is involved in development and progression of breast cancer, especially in invasion and metastasis.

Materials and methods

Tissue samples and cell lines

A breast cancer cell line, MCF7, was obtained from the Human Science Research Resources Bank (Osaka, Japan). Four breast cancer cell lines (SK-BR-3, MDA-MB-361, MDA-MB-231, and BT474) were obtained from the American Type Culture Collection (Manassas, VA, USA). MDA-MB-453, another breast cancer cell line, was obtained from the RIKEN Cell Bank (Tsukuba, Japan). These cells were cultured according to the suppliers' recommendations. A series of 67 primary invasive breast cancers without systemic drug therapy before surgery were obtained from patients who underwent surgery at the Department of Breast and Endocrine Surgery of Juntendo University, Tokyo during the period 2006–2010. These included 65 invasive ductal carcinomas (8 papillotubular, 21 solid-tubular, and 36 scirrhous carcinomas) and 2 invasive lobular carcinomas. In 39 of 67 tumors, both invasive and noninvasive lesions were present within the same specimen. Informed consent was obtained from patients before surgery for specimens to be used for this research. As summarized in Table 1, the average age of the patients at diagnosis was 56.9 years, ranging from 32 to 82 years. Of 67 patients, 24 patients were under 50 years of age, while 43 patients were over 50 years of age at time of diagnosis. Twenty-one cases had lymph node metastasis. Seventeen cases had lymphovascular invasion. Fifty-six cases had positive ER and/or PgR hormone receptor expression. Ten cases had Her2 overexpression. Twenty-three, 37, and 7 cases were diagnosed as nuclear grade 1, 2, and 3, respectively, whereas 31, 33, and 3 cases were diagnosed as pT stage 1, 2, and 3, respectively. Twenty-three cases were categorized as pathological stage I, 30 cases as stage IIA, 10 cases as stage IIB, and 4 cases as stage III. Fifty of 67 tumors with diameter equal to or greater than 1.5 cm were obtained surgically, and tissue samples were frozen immediately and stored at -80°C . This study was approved by the ethics committees of Juntendo University and the Institute of Medical Science, The University of Tokyo. All samples were diagnosed and classified according to the World Health Organization (WHO) grading system and the General Rules for Clinical